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14. ABSTRACT
Inflammation and edema are associated with respiratory and cutaneous exposure to sulfur mustard (SM) and with phosgene-induced lung injury. IL-8, a key chemotactic inflammatory cytokine, recruits neutrophils and contributes to progression of acute lung injury caused by inhalation of these chemical agents. We exposed human lung small airway cell (SAC) cultures to SM 25 to 400 mM or phosgene 0.1 to 6.4 ppm · min. IL-8 was increased after exposure to either agent. In SAC cultures exposed to SM (100 mM) and phosgene (1.6 ppm · min), IL-8 was increased above controls by 1013 +123 pg/ml and 965 +181 pg/ml, respectively. Exposure to higher concentrations of either agent increased cytotoxicity and reduced IL-8 towards levels observed in unexposed control SAC. Ibuprofen has shown efficacy against phosgene pulmonary toxicity in mice. Ibuprofen (62, 125, 250, 500, 1000 mM) significantly diminished phosgene-increased IL-8 in SAC cultures exposed to 2 ppm · min phosgene. Maximum inhibition of nearly 50% of phosgene-increased IL-8 was seen at 125 and 250 mM ibuprofen (from 1141 +143 pg/ml to 628 +105, 593 +69 pg/ml respectively). Chemical insult-increased IL-8 in SAC cultures provides an assay for screening countermeasures against the inhalation toxicity of chemical threat agents.

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sulfur mustard, phosgene, inhalation toxicity, drug screen

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ABSTRACT

Inflammation and edema are associated with respiratory and cutaneous exposure to sulfur mustard (SM) as well as with phosgene-induced lung injury. IL-8 is a key chemotactic inflammatory cytokine that recruits neutrophils and contributes to progression of acute lung injury caused by inhalation of these chemical agents. In the present study, human lung small airway cell (SAC) cultures were exposed to either SM 25 to 400 μM or phosgene 0.1 to 6.4 ppm • min. IL-8 was increased after exposure to either SM or phosgene. In SAC cultures exposed to SM (100 μM) and phosgene (1.6 ppm • min), IL-8 was increased above controls by 1013 ±123 pg/ml and 965 ±181 pg/ml, respectively. Exposure to higher concentrations of either agent increased cytotoxicity and reduced IL-8 towards levels observed in unexposed control SAC. Ibuprofen has shown efficacy against phosgene pulmonary toxicity in mice. Ibuprofen (62, 125, 250, 500, 1000 μM) significantly diminished phosgene-increased IL-8 in SAC cultures exposed to 2 ppm • min phosgene. Maximum inhibition of nearly 50% of phosgene-increased IL-8 was seen at 125 and 250 μM doses of ibuprofen (from 1141 ±143 pg/ml to 628 ±105, 593 ±69 pg/ml respectively). Chemical insult-induced increased IL-8 in SAC cultures provides an assay for screening countermeasures against the inhalation toxicity of chemical threat agents.

INTRODUCTION

Inflammation and associated inflammatory molecules such as IL-8 are implicated in the toxicity of the blister agent sulfur mustard (2,2'-dichlorodiethyl sulfide, SM) and the edemagenic agent phosgene (carbonyl chloride) (1,2,3,4). Increased levels of the rodent homologues to IL-8, NAP-1 in the skin of rabbits exposed to SM and MIP-2 in lungs of mice following inhalation exposure to phosgene have been reported (5,6). The anti-inflammatory drug ibuprofen has shown efficacy for phosgene-induced lung injury in rodents (reviewed, 5). Drugs with demonstrated efficacy against SM toxicity in the mouse ear vesicant model (MEVM) predominantly have anti-inflammatory action and inhibit SM-increased IL-8 in human epidermal keratinocyte (HEK) cultures (reviewed 3,7,8).

SM–increased IL-8 in HEK cultures is a biomarker for cutaneous inflammation that has been used as an *in vitro* assay for screening anti-vesicant drugs (2,3,8). The mechanistic and drug screening precepts for cutaneous SM injury, ascribed to SM-increased IL-8 in HEK cultures, might be extended to SM- and phosgene-exposed SAC cultures. SM- and phosgene-increased IL-8 in SAC cultures could provide a biomarker for pulmonary inflammation associated with respiratory injury. SM- and phosgene-increased IL-8 in SAC cultures could further provide an *in vitro* drug-screening assay for countermeasures against inhalation of toxic chemicals. This would fulfill a critical need for predicting medical countermeasures against the inhalation toxicity of chemical agents.

METHODS

Reagents: Sulfur mustard (SM, 2,2'-dichlorodiethyl sulfide) >98% pure was obtained from the U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD. Human lung epithelial small airway cells (SAC) and small airway growth media (SAGM™) and growth factors contained in SAGM BulletKit® were purchased from CAMBREX, Walkersville, MD. Quantikine® Human IL-8 Immunoassay Kit was purchased from R&D Systems Inc., Minneapolis, MN.

Cell Culture: SAC were cultured at 37° C in a 5% CO_2 incubator using standard procedures provided by the supplier. Subculturing to 24-well tissue culture plates was done using CAMBREX trypsin-EDTA. SAC were cultured at 100% confluence for 24 hr prior to exposure to SM or phosgene. These conditions were selected because drugs screened for inhibition of SM-increased IL-8 in HEK cultured under these conditions seem to better reflect efficacy in the MEVM (Cowan, unpublished observation).

IL-8: IL-8 was assayed by the quantitative sandwich enzyme technique described in an R&D Systems Inc. (Minneapolis, MN) pamphlet (Quantikine® Human IL-8 Immunoassay).

Sulfur Mustard Exposure: SAC cultured at 100% confluence were exposed to 25 to 400 μM SM in 1 ml SAGM per well. The plates were maintained at room temperature in a fume hood for 1 hr to allow venting of volatile agent and then transferred to a 5% CO_2 incubator at 37° C for a total incubation time of 24 hr. The same SAGM was used for the

growth phase and confluent SAC culture. This resulted in accumulation of background levels of IL-8 in control cultures (1509 ± 128 pg/ml).

Phosgene Exposure: SAC cultured at 100% confluence were exposed to 0.1 to 6.4 ppm • min of phosgene. The SAGM culture medium was removed from the well and tape (TimeMed Labeling Systems, Inc., Burr Ridge, IL) placed over the tissue culture plate well. Phosgene (Matheson Tri-Gas Inc., Joliet, IL) was injected by syringe (Hamilton Company, Reno, Nevada) punctured through tape. The tape was removed 1 min after exposure to the phosgene gas, and 1 ml of media was added to the exposed cells. Phosgene was 10% phosgene, 90% nitrogen, certified mixture grade. Syringe was a Hamilton Gas Tight Syringe #1705 for 50 µl size with removable side port needle 22 gauge for 50 ul size. Exposures were done at room temperature in a fume hood (Kewaunee Scientific Corporation, Statesville, NC). Following exposure, cells were transferred to a 5% CO_2 incubator at 37° C for a total incubation time of 24 hr. The replacement of SAGM at the time of phosgene exposure resulted in insignificant accumulation of background levels of IL-8 in unexposed controls (31 ± 33 pg/ml).

Ibuprofen Preparation: Ibuprofen sodium salt (FW 228.3; Sigma-Aldrich Co., St. Louis, MO) was diluted in sterile preservative free 0.9% saline solution (AmTech Group, Inc., Phoenix Scientific, Inc., St. Joseph, MO). The pH of the drug was adjusted with 0.25M HCl and 1M NaOH to pH 7.2 and filtered with a StarLB 0.45 micron filter (Costar Corporation, Cambridge, MA). Ibuprofen was added to SAC cultures exposed 30 min previously to 2 ppm • min phosgene. Final drug concentrations of 62, 125, 250, 500 and 1000 µM were selected based on ibuprofen efficacy against phosgene toxicity in rodent *in vivo* studies (reviewed, 5).

Data Analysis: A one-way analysis of variance (ANOVA) was used for each group, SM, phosgene, and ibuprofen, to compare the doses. If there was a significant dose effect, then a Dunnett's test was used to compare doses with the control, 0 dose. Statistical significance was defined as $p<0.05$. Data are presented as means ± standard error of mean (SEM), N = 4.

RESULTS

The SAC negative control background IL-8 levels were significantly higher for SM exposures (1509 ± 128 pg/ml) compared with the phosgene exposures (31 ± 33 pg/ml) because of the different cell culture conditions described in Methods. Therefore, SM-increased IL-8 is expressed as total IL-8 minus the accumulated background, and phosgene-increased IL-8 as total IL-8.

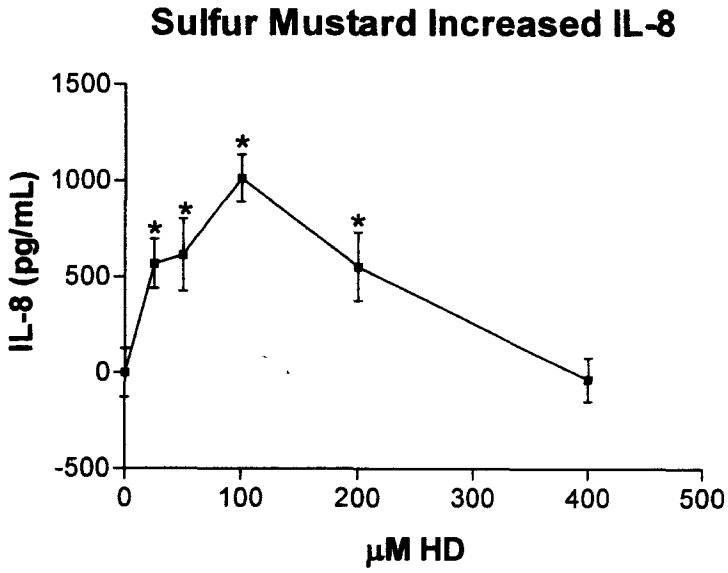

Figure 1. SM-increased IL-8 in SAC cultures. SAC cultures exposed 24 hr previously to 25, 50, 100, 200 or 400 μM SM demonstrated significant SM-increased IL-8 to all exposures except the 400 μM high dose. SM-increased IL-8 was calculated as total IL-8 pg/ml minus background control IL-8 (1509 ± 128 pg/ml). Results are presented as mean ± SEM, N= 4. Statistical significance* is defined as $p<0.05$ when compared with control.

SAC cultures exposed 24 hr previously to 25, 50, 100, 200 or 400 μM SM demonstrated significantly increased IL-8 to all exposures except the 400 μM high dose (Figure 1). The intermediate exposures of 100 μM SM gave maximum SM increased-IL-8 (1013 ±123 pg/ml) above background levels. High doses of 200 and 400 μM SM progressively increased cytotoxicity to > 90% and inhibited IL-8 toward unexposed control levels.

SAC cultures exposed 24 hr previously to 0.1 0.2. 0.4, 0.8, 1.6, 3.2 or 6.4 ppm • min phosgene for 1 min demonstrated significantly increased IL-8 at all exposures except 0.1 ppm • min. The intermediate exposure of 1.6 ppm • min phosgene gave the largest increases in IL-8 (965 ±181 pg/ml). Higher exposures at 3.2 and 6.4 ppm • min phosgene progressively increased cytotoxicity to > 95% and inhibited phosgene-increased IL-8 toward unexposed control levels (Figure 2).

The 1.6 ppm • min phosgene dose was rounded to 2 ppm • min for drug screening experiments. Ibuprofen significantly inhibited 2 ppm • min phosgene–increased IL-8 at all the tested doses. Maximum efficacy of nearly 50% inhibition was seen at the 125 and 250 μM doses (from 1141 ±143 pg/ml to 628 ±105, 593 ±69 pg/ml respectively, Figure 3).

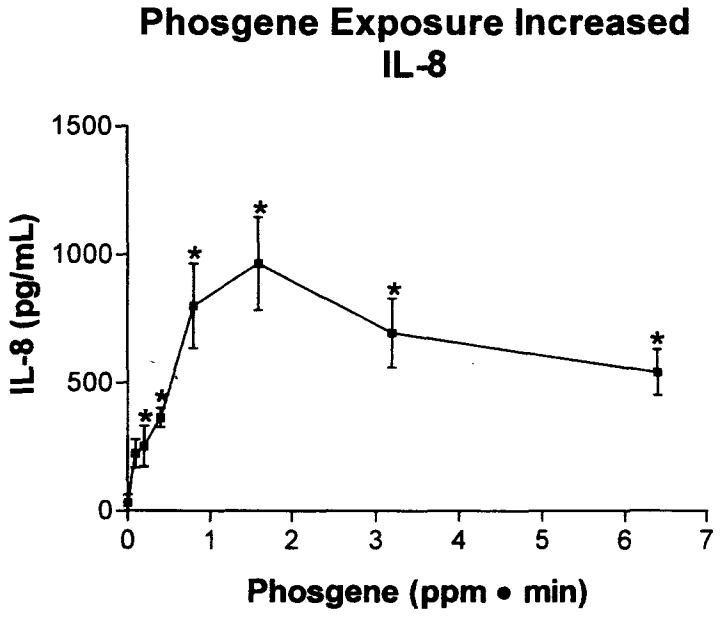

Figure. 2. Phosgene-increased IL-8 in SAC cultures. SAC cultures exposed 24 hr previously to 0.1 0.2. 0.4, 0.8, 1.6, 3.2 or 6.4 ppm • min phosgene demonstrated significantly increased IL-8 at all exposures except 0.1 ppm • min. Phosgene increased IL-8 was calculated as total IL-8 pg/ml. Results are presented as mean $\pm$ SEM, N= 4. Statistical significance* is defined as $p<0.05$ when compared with control.

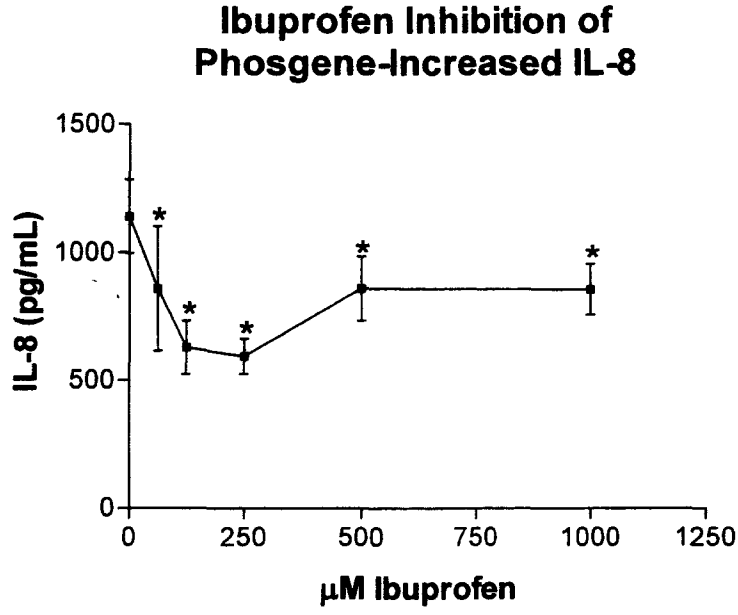

Figure 3. Ibuprofen inhibited phosgene-increased IL-8 in SAC cultures. Ibuprofen was added to SAC cultures exposed 30 min previously to 2 ppm • min phosgene. Final drug concentrations were 62, 125, 250, 500 and 1000 μM. Ibuprofen at all doses tested significantly inhibited phosgene–increased IL-8 at 24 hr after phosgene exposure. Phosgene-increased IL-8 was calculated as total IL-8 pg/ml. Results are presented as mean ± SEM, N= 4. Statistical significance* is defined as $p<0.05$.

Initially SAC seemed to be more sensitive to SM toxicity than previously reported for HEK. For example 200 μM SM caused slightly > 50% cytotoxicity in HEK cultures (9), whereas this dose caused > 90% cytotoxicity in SAC. However, in previous studies HEK cultures were 80-90% confluent (9), whereas in the current experiment SAC were cultured at 100% confluence for 24 hr HEK at 100% confluence for 24 hr were also more susceptible to SM cytotoxicity (unpublished observation).

Phosgene's cytotoxicity in SAC cultures was only slight (< 10%) at the 1.6 ppm • min exposure, which gave the maximum increased IL-8. However, phosgene toxicity was very pronounced (> 50%) at 3.2 ppm • min and almost total (> 95%) at 6.2 ppm • min phosgene. Interestingly, slight irregularities in the depth of the 24-well tissue culture plates and phosgene's density being greater than air produced higher exposures and greater cytotoxicity in slightly recessed areas of the plate. This resulted in a "bull's-eye" effect of trypan blue stained SAC with viable cells located at the periphery and center of the plate. This effect was very sight at 1.6 ppm • min and very pronounced at 3.2 ppm • min, and only small areas of central and peripheral viable cells were seen at 6.2 ppm • min phosgene.

DISCUSSION

Human SAC cultures exposed to either SM 25 to 200 μM or phosgene 0.2 to 6.4 ppm • min demonstrated significantly increased IL-8. A maximum increase of about 1000 pg/ml IL-8 in SAC cultures exposed to either phosgene or SM was observed (Figures 1 and 2). The pattern of IL-8 response, increase to maximum levels followed by inhibition at higher cytotoxic doses, was similar for both agents. Ibuprofen (62, 125, 250, 500, 1000 μM) significantly diminished phosgene-increased IL-8 in SAC cultures exposed to 2 ppm • min phosgene (Figure 3). Furthermore, the doses of ibuprofen that decreased phosgene-increased IL-8 about 50% in SAC also inhibited SM-increased IL-8 in HEK cultures to about the same extent (unpublished observation).

The inflammatory actions of SM and efficacy of a preponderance of drugs with anti-inflammatory actions against SM toxicity strongly implicate inflammation as a major component of SM cutaneous toxicity (1,2,3,7,8,10,11). SM and the edemagenic agent phosgene increased IL-8 in SAC cultures similar to IL-8 increases previously reported in SM-exposed HEK. Therefore, some mechanistic and drug screening precepts established for cutaneous toxicity might be extended to inhalation toxicity. Increased IL-8 in SAC cultures as a biomarker for inflammation may reflect mechanisms of toxicity and further provide an *in vitro* screening assay for medical countermeasures against inhalation toxicity of chemical threat agents. Finally, drugs with efficacy against inflammatory pathology caused by one class of chemical warfare agent might also show multi-threat medical countermeasure action against inflammatory pathology cause by other classes of toxic agents (3).

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